Immunocontraceptive Vaccines for Control of Fertility in the European Red Fox *(Vulpes vulpes)*

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Abstract: This paper describes the strategies being employed in the development of an immunocontraceptive vaccine using sperm antigens, to control fox populations in Australia. It is proposed that such a vaccine will be delivered orally in a bait, thereby ultimately stimulating a mucosal immune response within the female reproductive tract. The eventual success in producing such a vaccine requires the identification of gamete antigens that cause immunological infertility, a detailed understanding of the reproductive immunology of foxes, and the selection of the most effective form of antigen delivery system.

Keywords: Sperm antigens, immunocontraception, mucosal immunity

Introduction

Immunocontraception potentially offers the most effective method for the management and long-term population control of vertebrate pest species. The idea of using fertility control for such purposes is not new. In the early 1960's, a number of investigators examined the use of chemical sterilants to limit the reproductive capacity of animal populations (Linhart 1964). None of these methods proved effective, probably because these chemicals lead to castration of the target species. Castration removes the source of the key sex hormones, and this effect has the potential to interfere with the normal social structure of a target population, an undesirable outcome that could lead to a breakdown in established social hierarchies and may result in compensatory breeding.

Immunocontraception for Feral Species

In 1992, the Cooperative Research Centre for the Biological Control of Vertebrate Pest Populations was established in Australia to explore alternative methods of fertility control based on the use of gamete antigens as immunogens. One of the species being targeted in this research is the European red fox *(Vulpes),* which is a major vertebrate pest in Australian responsible for the loss of many native species through predation.

Our approach to immunocontraceptive control of the fox involves developing a bait-delivered oral vaccine. In this paper, I will discuss the experimental approaches used in the development of such a vaccine for foxes. Specifically, the focus will be on the identification of sperm antigens as vaccine candidates, the immunological questions that need to be addressed, and the development of appropriate delivery systems.

Although these considerations are directed toward an application for the fox, many of the concepts are relevant for immunocontraception in other species. The wider concerns relating to species specificity, and the use of recombinant vaccines, will be covered in the paper by Tyndale–Biscoe in this proceedings.

Components of an Immunocontraceptive Vaccine for Feral Species

A successful contraceptive vaccine should (1) block fertilization or early embryonic development; (2) affect both sexes; (3) be species specific; (4) provoke a prolonged and sustained immune response; and (5) not interfere with the normal social function of the animal. In particular, an effective mechanism for transmitting the vaccine throughout the target population must be found. This mechanism must be cost effective to manufacture and administer and must not impose hazards to the environment. These caveats make the development of an immunocontraceptive vaccine for wild animals highly challenging.

Reproductive Studies

Sperm Antigens as Targets for Immunocontraception

Vaccines developed toward sperm antigens would probably be capable of inducing infertility in both males and females. Potentially, this characteristic has the advantages not only of rendering sperm within the male genital tract incapable of fertilization before entry into the female but also of inactivating sperm within

the female genital tract. The direct immunization of males and females with extracts of sperm or testis results in a significant inhibition of fertility (Menge and Naz 1988).

We have tested the antifertility effect of antibodies to sperm in a group of six female foxes (Bradley 1994). Necropsy results revealed that there were 21 ovulations in this group of foxes. In all females examined, no live fetuses were found; 13 of 21 oocytes had been fertilized and embryos implanted, but all failed as determined by the presence of embryonic resorption scars. Examination of uterine flushes failed to find any unfertilized or preimplantation embryos. It was concluded that immunization with sperm results in an immunological block to fertility and that sperm immunization has two effects, one at the embryonic level and the other during fertilization.

The results of this experiment are consistent with those previously reported on the effect of experimentally induced sperm antibodies on fertility (O'Rand 1977, Koyama et al. 1984). Many of these experiments found that anti-sperm antibodies appeared to exert their effect on fertility not at the level of fertilization but rather at later stages ranging from early blastocyst development to implantation (Menge and Naz 1988), suggesting that some antigens are shared between the sperm and the embryo. Immunization against such complex mixtures of antigens is not really a practical approach for vaccine development. Instead, the individual antigenic components capable of causing immunological contraception need to be identified. Indeed, a number of specific sperm proteins can impair fertility when used to immunize animals (Naz 1987, LeVan and Goldberg 1990).

The most common approach to sperm antigen identification and selection is the use of monoclonal antibodies to sperm protein of the species under study (Anderson et al. 1987). One of the best examples is the PH-20 protein originally identified with monoclonal antibodies to guinea pig sperm. Fertility trials have shown that male and female guinea pigs immunized with PH-20 become infertile (Primakoff et al. 1988). More recently, the PH-20 genes from a number of other species have been cloned, leading to the possibility that this antigen may have applicability as a vaccine target in other species.

Another sperm antigen of interest is SP-10. This has been designated as a "primary vaccine candidate" by the World Health Organization Task Force on Contraceptive Vaccines (Herr et al. 1990a and b). Antibodies to SP-10 inhibit the penetration of hamster eggs by human sperm, and trials in baboons currently in progress will assess the applicability of this antigen as an immunocontraceptive for humans. Recently, a homologue of SP-10 (called MSA-63) has been identified in the mouse and cloned (Liu et al. 1990). Furthermore, antibodies to MSA-63 have been shown to have a strong inhibitory effect on the in vitro fertilization of mouse ova, providing good support that this class of antigens is worthy of study as potential targets for immunocontraception.

Fox Sperm Antigens Currently Being Assessed for Use in an Immunocontraceptive Vaccine

A number of monoclonal antibodies have been developed to fox sperm antigens and used to clone the cognate genes from a fox testis cDNA library. One of these candidate antigens, FSA-1r, has been through fertility trial testing and found to have no effect on fertility. Other antigens are currently in the fertility trial phase of assessment.

Fox Acr.1 (Acrosomal Protein 1)

The FSA-Acr.1 protein is located within the acrosomal matrix of fox sperm, and it is first detected during spermatogenesis on the developing acrosome of round and elongating spermatids (Beaton et al. 1995). A monoclonal antibody to FSA-Acr.1 (FSA-10) was used to screen a fox testis cDNA library, and a cDNA clone was isolated. Database searches with the deduced amino acid sequence of FSA-Acr.1 revealed that the clone has high homology to both human and baboon sperm protein SP-10 and the mouse sperm protein, MSA-63. The region of highest homology is within the carboxyl terminus. Within the central portion of the open reading frame, the fox sequence

contains amino acid motifs that are absent from both the human and baboon SP-10 and mouse MSA-63 sequences. We have expressed the FSA-Acr.1 protein in vitro, and this protein is being assessed in fertility trials to determine whether or not antibodies to FSA-Acr.1 can impair fertility.

Fox LDH-C_4

Lactate dehydrogenase C_4 (LDH-C_4) is an intracellular sperm-specific enzyme a portion of which is located on the sperm flagella plasma membrane. A number of studies have previously demonstrated that, when purified LDH-C_4 is used to immunize either mice, rabbits, or baboons, fertility was reduced by 60 to 80 percent (LeVan and Goldberg 1990). Epitope mapping studies of mouse and human LDH-C_4 have identified an antigenic peptide within the N-terminal region of the open reading frame, from amino acids 5–19 (Millan et al. 1987). This sequence also has the greatest variation in sequence between different species. We have recently cloned a fox LDH-C_4 cDNA and have derived sequence information from the 5' region of the open reading frame. This research has enabled us to synthesize a peptide to this region that was subsequently conjugated to the tetanus toxoid protein as an immunogenic carrier protein. This peptide-protein conjugate has been used to immunize female foxes by the intra-Peyers' patch route, and the immune responses and the effects of this immunization on fox fertility are currently being measured.

Fox PH-20

Cloning of the guinea pig PH-20 revealed that it has homology at the protein level with bee venom hylauronidase (Gmachl and Kreil 1993). Hylauronidase enzymatic activity is present within the head of mammalian sperm, and it has been shown that PH-20 has hylauronidase enzymatic activity (Gmachl et al. 1993). We have isolated a cDNA from a fox testis cDNA library encoding PH-20, and partial sequence analysis indicates close homology to PH-20 antigens cloned from other species. The antifertility effects in foxes of PH-20 immunization will be assessed with the whole protein and with selected peptide sequences.

Production of Recombinant Antigens

The in vivo testing of candidate antigens requires the large-scale production of recombinant proteins. A number of commercially available protein expression systems exist for this purpose; however, the selection of the system for a particular purpose eventually depends on the properties of the antigen under study. For example, is the protein highly glycosylated, and how important is this for antigenicity? Or, is the protein composed of multiple subunits? These considerations all have bearing on the success and selection of any one expression system. We have extensively used the maltose binding protein (MBP) expression system for the production of recombinant sperm proteins. This system produces a fusion of MBP to the protein of interest. Following expression, the fusion product is purified by affinity chromatography to yield a hybrid protein. The MBP can be cleaved and purified from the target protein, but this has some drawbacks in that small amounts of MBP still contaminate the antigen preparations, and loss of antigen occurs at each purification step. Unfortunately, the selection of expression systems for production of recombinant proteins is still something of a trial-and-error procedure, and several systems may have to be evaluated before selection of one that suits the purpose of the antigen under study.

In Vivo Fertility Testing of Recombinant Sperm Antigens

Ideally, during the selection of monoclonal antibodies that identify candidate sperm vaccine antigens, part of the testing procedure should include the use of functional assay to determine the effect of these antibodies on either sperm–egg binding or fertilization in vitro. While such assays are readily available for some species, researchers are limited in the fox in the routine use of such assays by both the biology of foxes and the paucity of an in vitro fertilization assay system. Because the fox is a seasonal breeder, the availability of gametes for such studies is restricted to 2 months every year, a fact that severely restricts the opportunity for assays. There is one reported study of attempts to establish an in vitro fertilization system for foxes. But, unfortunately, this procedure is still in the

early experimental stages, and its routine application is not feasible at present (Farstad et al. 1993).

An alternative is to test the antifertility effects of candidate antigen(s) in vivo in female foxes. This approach is both time consuming and expensive, but it is highly informative. For example, we have routinely performed necropsies on the immunized foxes at about 40 days after mating to evaluate the biological implications of the immunization regime(s) on fox reproduction. At the same time, samples of reproductive-tract fluids are collected for assays of the immunoglobulin levels within each section of the tract. This approach provides information on both the immunology of the treatment and the in vivo effects on fertility.

Do We Use Proteins or Peptides as Vaccine Antigens?

Ultimately, the choice will have to be made as to whether a recombinant vaccine for immunocontraception is developed which contains the full target protein or an antigenic peptide. Decisions will have to be based on the considerations of the species specificity of the antigen and the ability of any selected peptide epitope to produce a sufficient immune response to block fertility. The success of ongoing research on the selection, design, and construction of peptide antigens will be vital for the future development of peptide-based vaccines.

Immune Responses to Gamete Antigens

Mucosal Immune Response in the Female Reproductive Tract

The use of a bait-delivered oral immunocontraceptive for foxes requires a detailed understanding of the processes involved in the induction, modulation, and duration of genital tract mucosal immunity. The immune responses within the female genital tract are similar to that observed at other mucosal sites. Vaginal and cervical secretions contain high levels of immunoglobulin A (IgA) that appear to be locally synthesized. It has previously been shown that the reproductive mucosal site is linked to the common mucosal system and that IgA plasma cells stimulated at a distant site, such as the gastrointestinal tract, can rapidly migrate to the female reproductive tract (McDermott et al. 1980, Parr and Parr 1989 and 1990).

Using a model recombinant-derived antigen, maltose binding protein (MBP), we have investigated different immunization regimes to determine which route induces reproductive-tract mucosal immune responses within female foxes. Direct administration of antigen into the Peyers' patches (IPP) has been used because this route effectively mimics the oral presentation of an antigen to the gut associated lymphoid tissue (Dunkley and Husband 1990). Peyers' patch immunization induces a mucosal immune response within the female reproductive tract, but in the absence of a booster immunization, the antibody responses are transitory, particularly for IgA (fig. 1). This fact indicates that maintenance of vaginal IgA antibodies may require the antigen to persist.

To examine this, an experiment was conducted to test the effect of secondary immunizations with MBP on the maintenance of vaginal antibody levels. Three female foxes were immunized IPP and then boosted intradermally (ID) 1 and 2 months later. The results show that the levels of both serum and vaginal antibodies were maintained at higher levels and for longer compared with foxes receiving a single Peyers' patch immunization (fig. 2). In light of this information, female foxes in fertility trials are now routinely immunized IPP followed by a secondary immunization ID, thus ensuring that high antibodies levels are present within the reproductive tract throughout the period of the fertility trial.

Immune Responses and the Endocrine System

A number of studies have demonstrated that the sex hormones estrogen and progesterone can influence immunoglobulin G (IgG) and IgA antibody production within the female reproductive tract (McDermott et al. 1980). Any immunocontraceptive vaccine strategy will have to address the effect that these localized

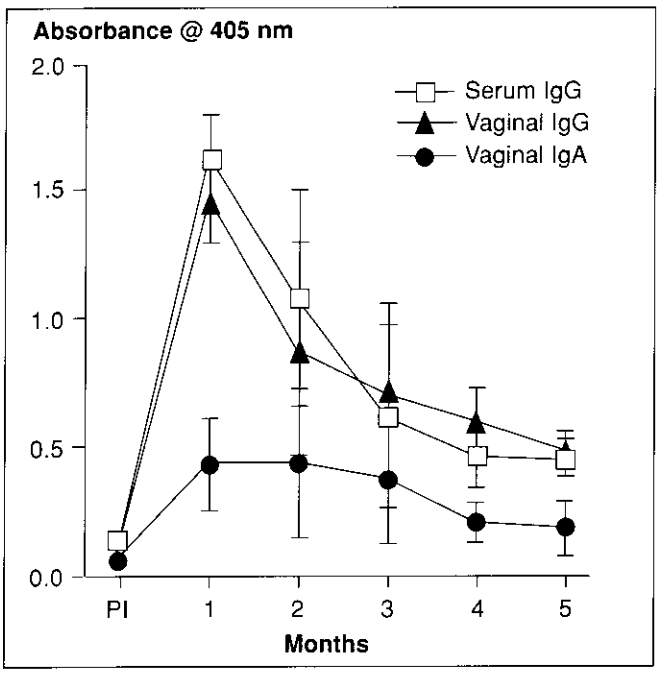

Figure 1. Serum and vaginal fluid antibody responses (Mean ± SD; $N = 3$) in female foxes as determined by enzyme-linked immunosorbent assay (ELISA). Each fox was immunized once with MBP into four Peyer's patches.

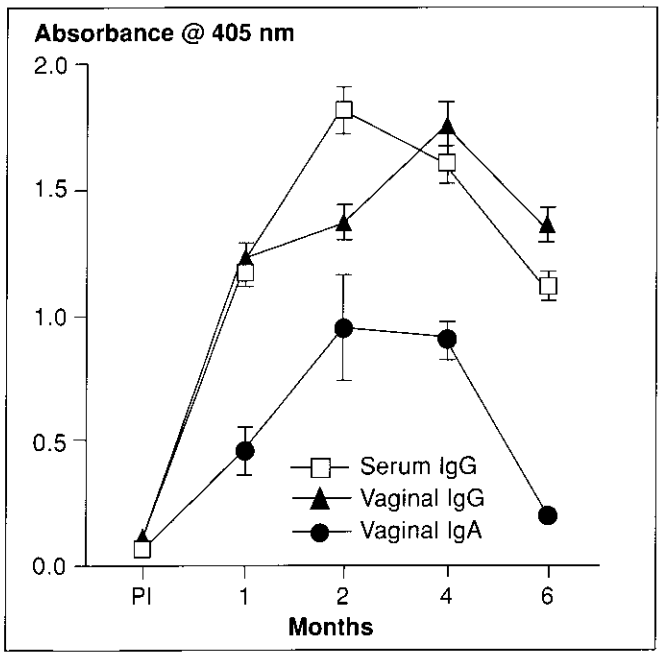

Figure 2. Serum and vaginal fluid antibody responses (Mean ± SD; $N = 3$) in female foxes as determined by ELISA after immunization with MBP into four Peyer's patches, followed by intradermal booster immunizations 1 and 2 months later.

changes in the immune status of females may have on immunocontraception. Effective immunocontraception will depend on the maintenance of high levels of antisperm antibodies within the oviducts, uterus, and vagina during mating. If changes in the localized antibody concentrations are substantial during this critical period, then contraception may be compromised (Wira and Sandoe 1987). Studies are under way in the fox to determine if localized changes are seen in the reproductive tract IgG and IgA during estrus.

Long-Term Maintenance of an Immune Response

A problem that may need to be addressed in the administration of an immunocontraceptive vaccine to an outbred fox population is the variability of the immune response between individuals. Effective application of a vaccine for fertility control requires that a high level of immunity be achieved among individuals exposed to the vaccine. It may, therefore, be necessary to include multiple antigenic determinants within a vaccine to stimulate a broad range of immune responses. In addition, the antigen(s) may need to be presented in conjunction with other highly immunogenic carrier proteins to maintain a contraceptive level of immunity.

Antigen Delivery Systems for a Fox Immunocontraceptive Vaccine

At present, three different vaccine delivery systems are being assessed to determine which will be the most effective for inclusion into the bait: (1) recombinant derived gamete antigen(s) encapsulated within microspheres, (2) a recombinant vaccinia virus capable of expressing foreign antigen(s) in the infected host, and (3) selected recombinant bacterial vectors such as the attenuated aroA strains of *Salmonella typhimurium.*

Microencapsulated Antigens

The effective oral presentation of antigens to the lower gastrointestinal tract is hampered by the degradation of protein within the stomach. A convenient way to overcome this is to use biodegradable microspheres that contain the entrapped antigen. These could be ultimately packaged within a bait, providing an effective oral delivery system whereby the vaccine antigen is delivered directly to the gut. The microspheres are taken up by the mucosae with the subsequent induction of a mucosal immune response (McGhee et al. 1992). In recent years, a number of investigators have reported the successful application of this technique for the delivery of antigens and the subsequent generation of mucosal immunity to the encapsulated antigens (Mestecky and Eldridge 1991).

We have recently completed a study to evaluate the efficacy of microspheres containing a recombinant sperm antigen to stimulate a mucosal immune response in rats (Muir et al. 1994). Microspheres were synthesized using the poly-DL-lactide-co-glycolide copolymer incorporating a recombinant source of the fox sperm protein FSA-1r (Bradley 1994). The oral administration of FSA-1r-loaded microspheres to rats resulted in a significant production of cells within the jejunum that were secreting IgA antibodies specific for the FSA-1r antigen. The level of stimulation was comparable to that obtained by either direct immunization of the Peyers' patches with microspheres containing antigen or unencapsulated antigen. These preliminary results indicate that further experiments would be worth pursuing to assess the utility of this approach for antigen delivery to foxes.

Viral Vectors

The use of recombinant vaccinia viral vectors containing the genes encoding selected sperm antigens may offer an excellent delivery system for an immunocontraceptive vaccine. For example, effective vaccination of foxes with a recombinant vaccinia expressing the rabies glycoprotein gene has proved enormously effective for immunizing foxes against rabies (Brochier et al. 1990). Building on these experiences, we are developing vaccinia vectors for application in immunocontraception. Such a system would allow further studies on the enhancement of the mucosal immunity, possibly by constructing vectors that coexpress IgA-specific stimulating cytokines (Ramsay et al. 1994).

A preliminary assessment of the immunological responses in foxes to the oral administration of a recombinant vaccinia viral vector expressing the hemagglutinin antigen (HA) has begun, and the results of these experiments will provide a basis for further experiments designed to test the utility of using a recombinant poxviruses for the delivery of immunocontraceptive antigens.

Bacterial Vectors

Recombinant bacterial vectors are an alternative delivery vehicle for a variety of vaccine antigens (Schodel 1992). The use of attenuated strains of *Salmonella typhimurium* as a live vector would be particularly applicable for stimulating reproductive tract mucosal immunity because *Salmonella* sp. colonize the intestinal tract and proliferate in the gut associated lymphoid tissue (Curtiss et al. 1989).

The use of selected mutant strains of *Salmonella* sp. has the advantage that they can be made avirulent without decreasing immunogenicity and are not infective outside the host. These considerations are important for any recombinant vaccine being considered for environmental release. However, a potential drawback is that immunity toward the carrier organism could develop, making subsequent exposure to the vaccine ineffective.

We have conducted an experiment to assess whether the attenuated *Salmonella typhimurium* aroA mutant strain is capable of stimulating mucosal immune responses in foxes after oral administration. Such information is a prerequisite to any future use of this organism as a vaccine vector. We have found that foxes given oral doses of *Salmonella typhimurium* readily produce serum immunoglobulin M (IgM) and IgG, and vaginal IgG and IgA antibodies to *Salmonella typhimurium* lipopolysaccharide over a 6-week period. The results indicate that foxes can respond immunologically to a single oral dose of *Salmonella*

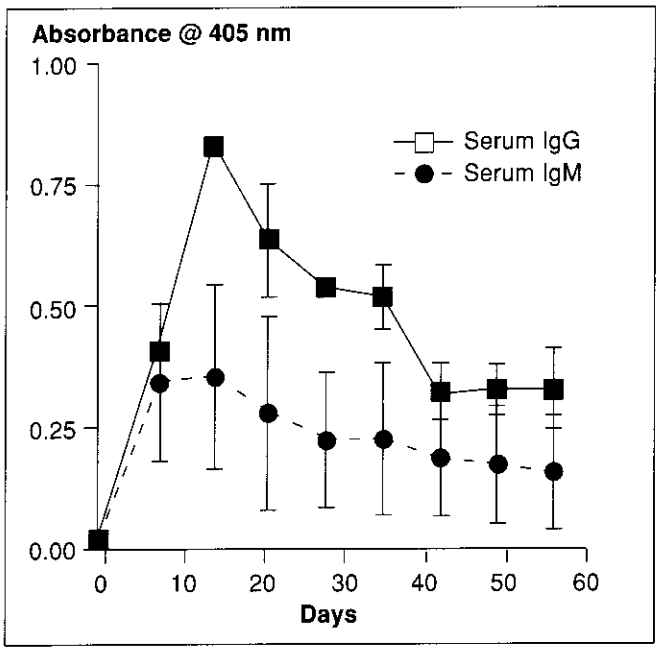

Figure 3. Serum IgG and IgM antibody responses (Mean ± SD; $N = 2$) in female foxes to lipopolysaccharide antigen after one oral dose of 5×10^9 *Salmonella typhimurium* (aroA mutant strain SL3261).

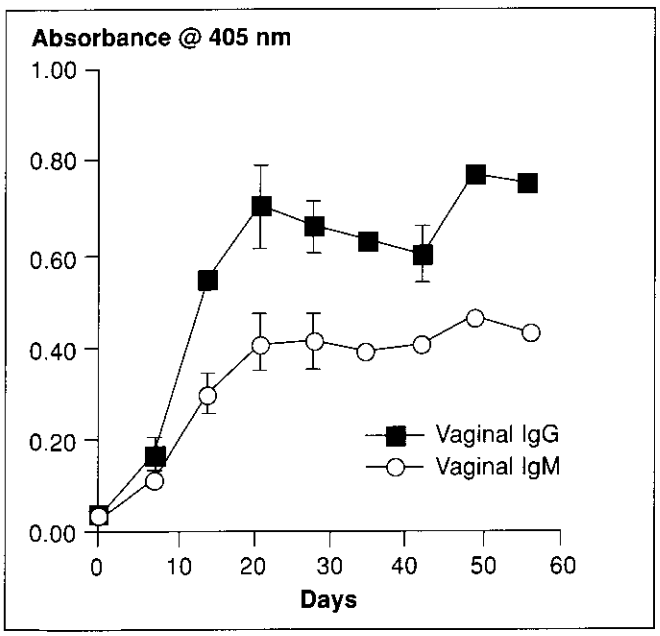

Figure 4. Vaginal IgG and IgA antibody responses (Mean ± SD; $N = 3$) in female foxes to lipopolysaccharide antigen after one oral dose of 5×10^9 *Salmonella typhimurium* (aroA mutant strain SL3261).

typhimurium that is sufficient to produce a high and sustained level of immunity within the female reproductive tract, albeit to a highly immunogenic antigen (figs. 3 and 4). Experiments are now in progress to construct a recombinant *Salmonella typhimurium* capable of expressing selected sperm antigens. Such recombinants will be screened for their ability to induce specific reproductive tract immune responses to the foreign antigen.

Concluding Remarks

Fertility control holds exciting prospects for the future management of wildlife populations. Internationally, a growing number of scientists and wildlife managers regard this approach as the only acceptable future method of managing wildlife populations. However, the obstacles that will be encountered in the development and implementation of such a technology are substantial. If the effort is successful, the rewards will be substantial, too. Each species will yield its own set of unique challenges.

In this overview, I have summarized key aspects relating to the reproductive and immunological studies that are required in the process of developing an immunocontraceptive vaccine for a vertebrate pest species. I have attempted to address, albeit in a rather brief way, the major considerations that need to be taken in to account when contemplating the development of a fertility control vaccine for wildlife. I have not considered the wider ecological implications of fertility control being imposed on a wildlife population. Such studies pose a whole new set of questions and challenges, and any project concerned with fertility control of wildlife will require a large, integral ecology research program to match the other facets of the work. Eventually, it will be the ecological studies that will assess both the impact and long-term consequences of fertility control on a particular wildlife population.

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